ACCUMULATION OF DRUGS INTO LIPOSOMES BY A PROTON GRADIENT

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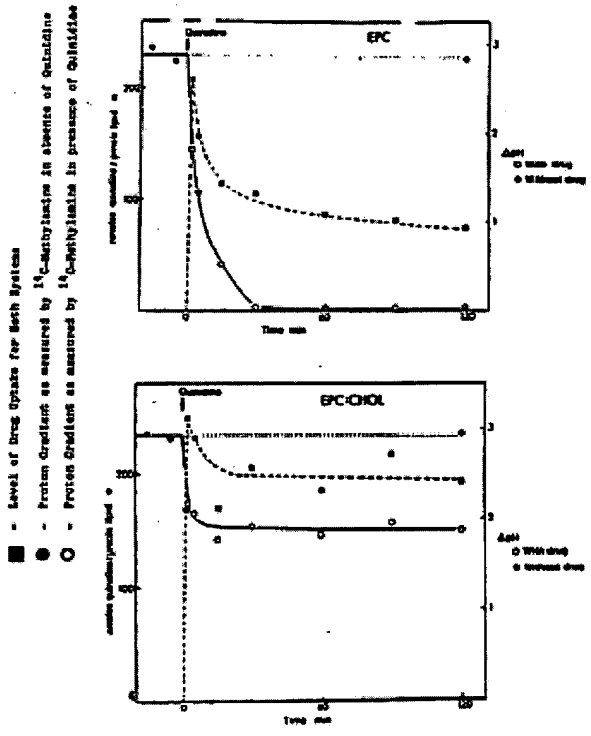
Abstract of **CA2056435**

2056435 9014105 PCTABS00002 The present invention relates to pharmaceutical compositions and methods of making liposome containing compositions exhibiting characteristics of great uptake. This uptake may be greater than what would be expected by the relationship defined by the Henderson-Hasselbach equation. The present invention also relates to liposomal compositions wherein the liposome comprises in part a membrane-stabilizing component, for example, cholesterol, which exhibits favorable characteristics in preventing rapid release of a pharmaceutical agent selected from the group consisting of quinine, quinidine and diphenhydramine after it has been formulated in liposomes. The present invention also relates to novel liposomal compositions comprising the bronchodilators metaproterenol, isoproterenol and terbutaline. The present invention also relates to minimum buffering capacity required to achieve liposomal encapsulation of pharmaceutical agents with maintenance of a major portion of the initial pH gradient.



ACCUMULATION OF DRUGS INTO LIPOSOMES BY A PROTON GRADIENT

Description of corresponding document: WO9014105

Accumulation of Druas into LiDosomes Bv a Proton Gradient
Field of the Invention
The present invention relates to pharmaceutical compositions and methods of making liposome containing compositions which exhibit characteristics of uptake which may be greater than expected by the relationship defined by the Henderson-Hasselbach equation.

The present invention also relates to liposomal compositions wherein the liposome comprises in part a membranestabilizing component, for example, cholesterol, which exhibits favorable characteristics in preventing rapid release of a pharmaceutical agent after it has been formulated in liposomes.

The present invention also relates to novel sustained release liposomal compositions comprising the bronchodilators metaproterenol, isoproterenol and terbutaline.

The present invention also relates to minimum buffering capacity required to achieve liposomal encapsulation of phrmaceutical agents with maintenance of a major portion of the initial pH gradient.

Backaround of the Invention
The therapeutic properties of many drugs may be dramatically improved by the administration in a liposomally encapsulated form [See, for example P.N. Shek and R.F. Barber, Mod. Med.

Canada, 41, 314-382,(1986)1. In certain cases, for example, in the administration of amphotericin B and doxorubicin [Lopez
Bernstein, et al., J. Infect. Dis., 151, 704-710, (1985) and Rahman, et al., Cancer Res., 40, 1532(1980)) toxicity is reduced while efficacy is maintained or even increased. The benefit obtained from liposomally encapsulated agents may be fortuitous and likely results from the altered pharmacokinetics and biodistribution of the entrapped drug[Ostro, et al., Amer. J.

Host. Pharm., in press.

The pharmacokinetics and biodistribution of an entrapped drug will largely depend on the character of the carrier system.

Optimization of a lipsomal drug requires an examination of a number of variables, including vesicle size, lipid composition and drug to lipid ratio. Most drug loading protocols, however, do not permit the independent variation of these parameters. Drugs which are passively trapped in liposomes will exhibit different drug to lipid ratios as the liposome size is varied due to changes in the trapped volume.

Several biogenic amines and antineoplastic agents have been shown to accumulate in liposomes in response to an imposed proton gradient known as "remote loading" [See, for example
Mayer, et al., Biochim.Biorhvs. Acta, 857, 123, (1986), Mayer, et al., Biochemistry, 27, 2053, (1988) and M.B. Bally, et al.,
Chem.Phvs. Lipids, 47, 97, (1988)]. This loading technique allows independent variation of any of the liposomal parameters.

Much higher drug to lipid ratios can be achieved in comparison to conventional techniquestMayer, et al. Chem. Phvs. Lipids, 40, 333 (1986)]. In addition, the transmembrane distribution of the drug is generally determined by the proton gradient which modulates drug leakage by changes in the buffering capacity of the intravesicular medium. The use of proton and other ion gradients to trap drugs which are non-zwitterionic weak bases has been shown to be practical for adriamycin, the local anaesthetics dibucaine and dopamine and other drugs. Advantages of this system include efficient drug trapping, slower rates of drug release than passively trapped drug, and higher drug to lipid ratios than can otherwise be achieved.In addition, because the liposomes can be prepared in the absence of the drug, problems with drug release during storage, or drug degradation during liposomal preparation can be avoided.

Intraliposomal drug accumulation in response to pH gradients is believed to occur in a manner similar to that of other weak bases, for example, the pH gradient probe methylamine.

Methylamine equilibrates across liposomal membranes in the uncharged form, and re-ionizes according to

the Henderson
Hasselbach relationship of the pH of its environment. The equilibrium distribution reflects the transmembrane pH gradient, and its redistribution can be used to measure these gradients.

However, not all pharmaceutical agents which possess the capacity to be ionized according to Henderson-Hasselbach relationships accumulate in liposomes according to this relationship.

In fact, certain agents do not seem to accumulate at all. In addition, certain agents which may accumulate according to this relationship immediately undergo release, resulting in unworkable pharmaceutical formulations which must be used immediately after production and which are virtually unusable as sustained release products.

Liposomes are completely closed lipid bilayer membranes which contain entrapped aqueous volume. Liposomes are vesicles which may be unilamellar (single membrane) or multilammelar (onion-like structures characterized by multiple membrane bilayers, each separated from the next by an aqueous layer). The bilayer is composed of two lipid monolayers having a hydrophobic"tail" region and a hydrophilic"head region. In the membrane bilayer, the hydrophobic (nonpolar) "tails" of the lipid monolayers orient toward the center of the bilayer, whereas the hydrophilic (polar) "heads" orient toward the aqueous phase. The basic structure of liposomes may be made by a variety of techniques known in the art.

Liposomal encapsulation could potentially provide numerous beneficial effects for a wide variety of pharmaceutical agents and the remote loading technique should prove instrumental in realizing the potential of liposomally encapsulated agents.

In addition, a high trapping efficiency for loading liposomes results in very little drug being lost during the encapsulation process, an advantage that proves to be important when dealing with expensive drugs. However, the use of liposomes to administer drugs has raised problems with regard both to drug encapsulation and drug release during therapy. For example, even with the present use of "remote loading" systems, there is a continuing need to increase trapping efficiencies so as to minimize the lipid load presented to the patient. Secondly, even with increased trapping efficiencies, there is no guarantee that the release characteristics of the loaded liposome will reflect acceptable sustained release characteristics. Many drugs have been found to be rapidly released from liposomes after encapsulation. Such release reduces the beneficial effects of liposomal encapsulation.

Obiects of the Present Invention
It is an object of the present invention to provide novel liposomal compositions and general methods for making such compositions which are designed to maximize the uptake of a pharmaceutical agent into the liposome, thereby increasing the amount of drug which can be loaded into liposomes and decreasing the lipid load presented to the patient during administration of liposomes.

It is a further object of the present invention to provide novel liposomal compositions and methods for making such compositions which prevent the rapid disadvantageous release of pharmaceutical agent before administration of the liposomes.

It is a further object of the present invention to provide novel brochodilator liposomal compositions.

BriefDescriction ofthe Fiaures
Figure 1 shows the accumulation of mitoxantrone by EPC vesicles exhibiting a proton gradient with an internal aqueous buffer system comprising 300 mM citrate, pH 4.0 and an external buffer system comprising 300 mM NaC1, 20 mM HEPES, pH 7.5.

Accumulation was rapid and complete and evidenced no release over several hours.

Figure 2A shows the response of timolol uptake in EPC vesicles (partial accumulation, uptake stable). The level of uptake is about 100 nmoles/umole (about 50% of available drug).

Figure 2B shows the response of quinacrine, which is similar to timolol in EPC vesicles. The level of uptake is about 80 nmoles/umole lipid after 30 minutes.

Figure 3A shows the response of quinidine uptake in EPC vesicles (complete accumulation, rapid release). Within 30 minutes about 50% of the drug leaks back out of the vesicles.

Figure 3B shows the effect of added cholesterol to the uptake of quinidine and the stability of the pH gradient.

Figure 4 shows the effect of physostigmine to transmembrane pH gradient. Under the conditions used to assess drug uptake (200 uM physostigmine) only a small decrease in the measured pH is observed.

Figure 5 shows the entrapment of metaproterenol, terbutaline and isoproterenol in response to pH gradients using egg phosphatidylcholine 200 nm extruded liposomes.

Figure 6 demonstrates the effect of drug uptake on the residual pH gradient as measured by methylamine redistribution in the presence and absence of isoproterenol. As shown, when the internal and external pH is 7.4 or 4.0 (no gradient), the methylamine does not detect any pH gradient.

Figure 7 shows the effect of temperature on the Entrapment of Metaproterenol in response to pH gradients at21eC, 37"C and60'C,
Figure 8 shows the influence of cholesterol on the accumulation of metaproterenol in response to a pH gradient.

Figure 9 shows the influence of varying the external drug concentration on the level of metaproterenol uptake.

Figure 10 shows the effect of internal buffering capacity on drug uptake.

Summarv of the Invention
The present invention relates to liposomal compositions having a pH gradient which exhibit markedly increased accumula tion of pharmaceutical agents above that expected from the Henderson-Hasselbach relationship by formulating the liposomes utilizing a first internal aqueous buffer and a second external aqueous buffer wherein the concentration of the pharmaceutical agent exceeds its solubility product in the internal buffer following uptake. Therefore, preferably, the pharmaceutical agent exhibits a solubility within the liposome which is less than the final concentration of agent within the liposome. Preferably, the solubility of the pharmaceutical agent is less than about 20 mM and most preferably less than about 10 mM.In addition, the internal buffer solution has a buffer strength of at least about 50 mMol, preferably about 100 to about 300 mMol and most preferably about 300.

The present invention also relates to liposomal compositions comprising in part, membrane-stabilizing components, for example, cholesterol, among other lipids, to prevent the rapid release of certain pharmaceutical agents from liposomes which do not contain the membrane-stabilizing components. Such liposomes preferably comprise a mixture of phosphatidylcholine and cholesterol in a molar weight ratio of about 55:45.

The present invention also relates to liposomal compositions having a pH gradient containing bronchodilators selected from the group consisting of metaproterenol, terbutaline and isoproterenol. It has been shown that the above agents, which heretofore have not been formulated in such liposomes, will accumulate into liposomes to an appreciable extent to produce effective, stable liposomal compositions useful for treating conditions requiring sustained release of bronchodilators. Such liposomal compositions comprising bronchodilator formulations may be useful for treating a number of conditions, including asthma.

Such compositions are expected to have a longer residence time in the lung than the same free drug, thus obtaining concentrations of bronchodilator at the site of activity within the lung for a period longer than for compositions presently available. Such compositions may be formulated as aerosols within a pharmaceutically acceptable solution for administration of bronchodilators directly into the lungs for treatment of acute asthma attacks.

The brochodilator compositions of the present invention have been shown to effectively accumulate in liposomes comprising eggphosphatidylcholine (EPC) as well as a mixture of phosphatidylcholine and cholesterol (55:45, w:w). The liposomes comprising the mixture of phosphatidylcholine and cholesterol, accumulate the bronchodilator to about the same relative extent as the EPC liposomes, although the time need for accumulation is longer for the cholesterol containing liposomes
In general, the liposome compositions of the present invention have a drug to lipid molar ratio ranging from about 0.5% up to about 50%.The liposomes of the present invention may comprise phospholipids such as egg phosphatidylcholine (EPC), hydrogenated soy phosphatidylcholine, distearoylphosphatidylcholine, dimyristoylphosphatidylcholine, or diarachidonoylphosphatidylcholine, among others, and may additionally comprise a number of steroidal compositions, as well as other compositions.

In general, the liposomes range in size from about 0.05 to greater than 2 microns, with a preferred range being about 0.05 to about 0.3 microns. Most preferably, the liposomes are unilamellar and range in size

from about 0.1 to about 0.3 microns. These unilamellar liposomes may be homogeneous or unimodal with regard to size distribution.

The liposomes of the present invention may be administered via oral, parenteral, buccal, topical, and transdermal routes of administration, among other routes of administration.

Detailed Description of the Invention
The present invention utilizes efficient trapping of pharmaceutical agents in liposomes exhibiting a transmembrane ionic gradient, preferably a transmembrane pH gradient, which can result in an accumulation of the agent in an amount significantly higher than otherwise expected from the Henderson-Hasselbach relationship. Liposome compositions of the present invention comprise at least one lipid, a phrmaceutical agent accumulated therein, an internal buffer solution wherein the solubility of the pharmaceutical agent within the buffer solution is less than the concentration of the agent within the liposome and an external buffer solution wherein the solubility of the pharmaceutical agent is preferably at least about 0.2 mM. As used throughout the specification, the terms pharmaceutical agent and drug are synonymous.

The liposomes of the present invention may be formed by any of the methods known in the art, but preferably they are formed according to the procedures disclosed in Balley, et al., PCT Application No. US86/01102, published February 27, 1986 and Mayer, et al. PCT Application No. US88/00646, published Sep tember 7, 1988. These techniques allow the loading of liposomes with ionizable pharmaceutical agents to achieve interior concentrations considerably greater than otherwise expected from the drugs' solubility in aqueous solution at neutral pH and/or concentrations greater than can be obtained by passive entrapment techniques. In this technique, a transmembrane ion (pH) gradient is created between the internal and external membranes of the liposomes and the pharmaceutical agent is loaded into the liposomes by means of the ion (pH) gradient, which drives the uptake.The transmembrane gradient is generated by creating a concentration gradient for one or more charged species, for example Na+,Cl-, K+, Li+,OH and preferably H+, across the liposome membranes, such that the ion gradient drives the uptake of ionizable pharmaceutical agents across the membranes. In the present invention, transmembrane ion (H+) gradients are preferably employed to produce the ion gradient and load the pharmaceutical agents, which tend to have weakly basic nitrogen groups, into the liposomes.

In the present invention, liposomes are preferably first formed in an aqueous buffer solution. The first solution is either acidic or basic, depending upon whether the pharmaceutical agent to be loaded produces a charged species at basic or acidic pH. For example, in the case of loading weakly basic pharmaceutical agents, a charged species is produced at low pH, i.e., a pH of about 2.0 to 5.0, preferably a pH of about 4.0.

After formation of liposomes having an acidic internal aqueous buffer solution, the buffer solution external to the liposomes is then modified to a pH significantly above the pH of the internal buffer solution, preferably at least about 3.0 to 4.0 pH units above the internal buffer solution. The modification of the external buffer results in a pH gradient which drives the accumulation of pharmaceutical agent within the liposome. The internal buffer solution may differ from the external buffer solution only in the difference in pH. In general, uncharged pharmaceutical agent will pass through the lipid layer(s) of the liposome much more readily than will charged (protonated, in the case of weakly basic pharmaceutical agents) agent.Thus, uncharged pharmaceutical agent in the external buffer will readily pass through the liposome into the internal buffer, become protonated, and remain within the liposome as a "trapped" protonated molecule which does not readily pass through the liposome layer(s). Pharmaceutical agent will thus concentrate in the liposome as a function of the pH gradient between the internal and external buffer solutions.

Such loading according to the above procedure, while effective for certain pharmaceutical agents, often does not result in maximum loading. Even if it is assumed that the pharmaceutical agent is maximally soluble in the internal and external buffer solutions and will readily pass through the liposomal layer(s) (an assumption not always borne out by reality), the maximum loading will generally reflect the relationship defined by the Henderson-Hasselbach equation[HA+]in/[HA+]out= [H+]in/[H+]out. However, a number of factors are believed to effect the ability of a pharmaceutical agent to accumulate.These factors include the partitioning of the unprotonated agent within the lipid layers(s), the difference in
PKa between protonated species that exist in the buffer solution and species associated with the membrane, the buffer capacity of the internal buffer solution and the solubility of the protonated species within the internal buffer.

It has now been determined that the most important factor influencing the accumulation of pharmaceutical agent within a liposome above what is expected from the Henderson-Hasselbach equation is the solubility of the protonated species of the agent within the internal buffer solution. It has been determined that the

solubility of the protonated species of the pharmaceutical agent to be accumulated will influence the level of uptake and accumulation which may be substantially greater than that predicted by the Henderson-Hasselbach equation.Liposome compositions which are formulated using an internal buffer solution in which an ionized pharmaceutical agent is minimally soluble and which preferably precipitates the ionized agent, will drive the accumulation of the pharmaceutical agent within the liposome beyond what would otherwise be expected to produce liposomes which consistently should have high trapping efficiencies approaching 100%.

In the present invention, it has surprisingly been discovered that the solubility of the pharmaceutical agent in the internal buffer may ultimately control the ability of the pharmaceutical agent to load into the liposome to an extent greater than that predicted by the Henderson/Hasselbach relationship. Thus, in the present invention, liposome compositions are preferred which are formed utilizing a first internal buffer solution of either basic (pH about 8 to 10) or acidic (pH about 3.0 to 5.0) character and a second external buffer solution, the pH of which is preferably between about 6.5 and 8.0, preferably 7.4. The high or low pH of the internal buffer relative to a neutral pH of the external buffer produces a transmembrane gradient which acts to drive the accumulation of the agent in the liposome.It has surprisingly been discovered that the most important factor in determining the ultimate amount of agent which may be loaded into liposomes using the transmembrane gradient to drive the accumulation of the agent into the liposomes above that expected by the Henderson-Hasselbach equation is the solubility of the agent in the internal buffer solution.

In general, internal buffer solutions useful in embodiments of the present invention are chosen so that the pharmaceutical agent to be accumulated has a solubility within the internal buffer solution which is less than the total agent to be accumulated in the liposome. Generally, the solubility of the pharmaceutical agent in the internal buffer solution is no greater than about 65 mM, preferably no greater than about 20 mM and most preferably no greater than about 10 mM.

The internal buffer solution is also chosen to maximize the buffer strength of the internal solution. It is believed that the buffer strength of the internal buffer solution is also important to the total accumulation of agent within the liposome and internal buffer solutions are chosen to maximize this strength. Of course, the solubility of the agent within the internal buffer solution is also a most important factor in determining accumulation. Therefore, where a buffer solution is to be chosen, it is both the solubility factor and the buffer strength factor which should be maximized in choosing useful buffer solutions. In the present invention, it has been determined that the buffer strength of the internal buffer solution should be at least about 50 mM, preferably about 100 mM to about 300 mM and most preferably about 300 mM.

Lipids which can be used in the liposome formulations of the present invention include synthetic or natural phospholipids and may include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol(PI), sphingomyelin(SPM) and cardiolipin, among others, either alone or in combination. The phospholipids useful in the present invention may also includedimyristoyl- phosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG). In other embodiments, distearylphosphatidylcholine (DSPC),dipalmitoylphosphatidylcholine (DPPC), orhydrogenated.

soy phosphatidylcholine (HSPC) may also be used. Dimyristoylphosphatidylcholine (DMPC) and diarachidonoylphosphatidylcholine (DAPC) may similarly be used. Due to the elevated transition temperaturres (Tc) of lipids such as DSPC (Tc of about65 C),
DPPC (Tc of about45it) and DAPC (Tc of about 85'C), such lipids are preferably heated to about their Tc or temperatures slightly higher, e.g., up to about5'C higher than the Tc, in order to make these liposomes. In preferred embodiments, egg phosphatidylcholine is used.

In a number of embodiments of the present invention, a steroidal component may be added to the liposome. Any of the above-mentioned phospholipids may be used in combination with at least one additional component selected from the group consisting of cholesterol, cholestanol, coprostanol or cholestane. In addition, polyethylene glycol derivatives of cholesterol (PEGcholesterols), as well as organic acid derivatives of sterols, for example cholesterol hemisuccinate (CHS) may also be used in combination with any of the above-mentioned phospholipids.

Organic acid derivatives of alpha-tocopherol hemisuccinate, (THS) may also be used. CHS- and THS-containing liposomes and their tris salt forms may generally be prepared by any method known in the art for preparing lipsomes containing sterols Any of the above-mentioned sterols may be used in liposomes, so long as the resultant phospholipid-sterol mixture yields stable liposomes.

In particular, see the procedures of Janoff, et al., U.S. Patent
No. 4,721,612, issued January 26, 1988, entitled "Steroidal

' Liposomes", and Janoff, et al., PCT Publication No. 87/02219, published April 23, 1987, entitled "Alpha Tocopherol-Based
Vehicles", relevant portions of which are incoporated by reference herein.

In certain embodiments in which the liposomes are designed to prevent rapid release of the pharmaceutical agent, cholesterol in an amount equal to about 30 mole% to about 45 mole% by weight of the lipid comprising the liposome is preferably used in combination with any of the above-named phospholipids or phospholipid/steroid combinations. Such compositions should, in general, prevent the undesired rapid release of accumulated pharmaceutical agent from the liposome. Any combination of membrane-stabilizing component and lipid may be used which prevents rapid release of pharmaceutical agents from the liposome, and one of ordinary skill in the art will be able to modify the membrane-stabilizing component and the phospholipid to formulate liposomes which prevent rapid release of the pharmaceutical agent.Most preferably, liposomes comprising a mixture of about 45 mole % by weight cholesterol and about 55 mole % by weight phosphatidylcholine are used in this aspect of the present invention. Although any number of pharmaceutical agents which show a proclivity to release rapidly from liposomes may be used in this aspect of the present invention, it has been determined that the agents quinine, diphenhydramine and quinidine are especially prone to rapidly release from liposomes and thus liposomal formulations comprising these agents preferably comprise cholesterol in an amount equal to about 30 to 45 mole % and preferably about 45 mole % of the lipid plus membrane-stabilizing component.Although it is difficult to determine, strictly on the basis of chemical structure, that a pharmaceutical agent will rapidly release from a liposomal formulation, one of ordinary skill in the art will be able to assess the degree of release of the agent and formulate a liposomal product consistent with the teachings of the present invention. As in other embodiments of the present invention, any buffer solution may be used for the internal and external buffer solutions in this aspect of the present invention regardless of the solubility of the pharmaceutical agent therein. However, the preferred buffer solutions are chosen so that the solubility of the pharmaceutical agent is less than the concentration of the agent within the liposome, preferably is less than 20mM and most preferably is less than 10 mM as is the case with other embodiments of the present invention.

Several methods may be used to form the liposomes of the present invention. For example, multilamellar vesicles (MLVs), stable plurilamellar vesicles (SPLVs), or reverse phase evaporation vesicles (REVs) may be used. Preferably, however, MLVs are extruded through filters forming large unilamellar vesicles(lugs) of sizes dependent upon the filter size utilized. In general, polycarbonate filters of 30, 50, 60, 100, 200 or 800 nm pores may be used. In this method, disclosed in Cullis, et al.,
PCT Publication Ho. WO 86/000238, January 16, 1986, relevant portions of which are incorporated by reference herein, the liposome suspension may be repeatedly passed through the extrusion device resulting in a population of liposomes of homogeneous size distribution.For example, the filtering may be performed through a straight-through membrane filter (a Nucleopore polycarbonate filter) or a tortuous path filter (e.g. a Nucleopore filter membrafil filter (mixed cellulose esters) of 0.1 um size), or by alternative size reduction techniques such as homogenization.

The size of the liposomes may vary from about 0.03 to above about 2 microns in diameter; preferably about 0.05 to 0.3 microns and most preferably about 0.1 to about 0.2 microns. The size range includes liposomes that are MLVs, SPLVs, orLugs. In the present invention, the preferred liposomes are those which are unilamellar liposomes of about 0.1 to about 0.2 microns.

As described hereinabove, a number of lipids may be used to form liposomes having a gel to liquid crystalline Tc above ambient temperature. In such cases, an extruder having a heating barrel or thermojacket may be employed. Such a device serves to increase the liposome suspension temperature allowing extrusion of the LWs. The lipids which are used with the thermojacketed extruder are, for example, DSPC, DPPC, DMPC and DAPC or mixtures thereof, which may include cholesterol in certain embodiments for preventing the rapid release of pharmaceutical agents from the liposome. Liposomes containing DSPC are generally extruded at about 65'C, DPPC at about 45'C and DAPC at about850C (about50c above the lipidTc).

As indicated, the preferred liposome for use in the present invention are LWs of about 0.06 to about 0.3 microns in size. As defined in the present application, a homogeneous population of vesicles is one comprising substantially the same size liposomes, and may have a Gaussian distribution of particle sizes. Such a population is said to be of uniform size distribution, and may be unimodal with respect to size. The term "unimodal" refers to a population having a narrow polydispersity of particle sizes, and the particles are of a single"mode".

A liposomal population is unimodal if, when measured by quasi elastic light scattering methods, the population approximates to a Gaussian distribution, and if a second order polynomial will fit the natural logarithm of the autocorrelation function of a sample (Koppel, 1972, J. Chem. Phys., 57:4814).

The closer this fit, the better the measure of unimodality. The closeness of this fit may be determined by how close the chi square (chi2) value of the sample is to unity. A chi2 value of 2.0 or less is indicative of a unimodal population.

Other size reduction techniques may be employed in practiing the present invention. For example, homgenization or mill ing techniques may successfully be employed. Such techniques may yield liposomes that are homogeneous or unimodal with regard to size distribution.

Liposomes may be prepared which encapsulate the first aqueous buffer solution having the characteristics described hereinabove. For a typical liposome preparation technique as fully described hereinabove, this first aqueous buffer solution will surround the liposomes as they are formed, resulting in the buffer solution being internal and external to the liposomes. To create the concentration gradient, the original external buffer solution may be acidified or basified so that the concentration of charged species differs from the internal buffer, or alternatively, the external buffer may be replaced by a new external medium having different charge species.The replacement of the external medium can be accomplished by various techniques, such as, by passing the liposome preparation through a gel filtration column, e.g., a Sephadex column, which has been equilibrated with the new medium, or by dialysis or related techniques.

During preparation of the liposomes, organic solvents may also be used to suspend the lipids. Suitable organic solvents for use in the present invention include those with a variety of polarities and dielectric properties, which solubilize the lipids, for example, chloroform, methanol, ethanol, dimethylsulfoxide (DMSO), methylene choloride, and solvent mixtures such as benzene:methanol (70:30), among others. As a result, solutions (mixtures in which the lipids and other components are uniformly distributed throughout) containing the lipids are formed. Solvents are generally chosen on the basis of their biocompatability, low toxicity, and solubilization abilities.

One preferred embodiment of the present invention is a 3 component liposomal-pharmaceutical agent treatment system which allows for highly efficient entrapment of the agent at the clinical site. When the pharmaceutical agent is one that loads in response to a transmembrane pH gradient where the interior of the liposome is acid, the first component of the system (Vial 1) comprises liposomes in an acidic buffer solution, in which for example, citric acid buffer (300 mmol., pH about 3.8 to 4.2, preferably 4.0) or another buffer in which the ionized form of the pharmaceutical agent to be trapped is only marginally soluble (solubility less than the final concentration of agent within the liposome, preferably no greater than about 20 mM and most preferably no greater than about 10 mM).The second component of the system (Vial 2) comprises a basic buffer solution, for example, a sodium carbonate or sodium bisphosphate solution at about 0.5 M, pH about 10 to 12, preferably about pH 11.5, which serves to become part of the external buffer solution of the liposome formulation. For purposes of maximizing the loading of the pharmaceutical agent within the liposomes it is preferable that the pharmaceutical agent has a solubility within the external buffer solution of at least about 0.2 mMol. The third component (Vial 3) is the pharmaceutical agent. The above-described treatment system may be provided as a 3-vial system, the first vial containing the liposomes in acidic medium, the second vial containing the base, and the third vial containing the pharmaceutical agent as described hereinabove.A similar treatment system may be provided for a pharmaceutical agent that loads in response to a transmembrane gradient wherein the internal buffer of the liposomes is relatively basic i.e., has a pH about 8.5-11.5. The first component comprises liposomes in a relatively basic buffer, for example, sodium carbonate or sodium bisphosphate, at a pH of about 8.0-11.0, preferably about 10. The second component comprises a relatively acidic or neutral solution as the external buffer for theliposo;rnes, for example, 150 mM NaClbuffer/150 mM
HEPES buffer at a pH of about 7.4. The third component comprises the pharmaceutical agent which is less ionized at the pH of the external buffer and is ionized-at the pH of the internal buffer.

Following the formation of the pH gradient across the liposomes (by admixing the first and second vials), the liposomes may be heated prior to admixing with the drug. Under certain circumstances, and in cases where the pharmaceutical agent is to be loaded into liposomes comprising at least about 30 mole % cholesterol to minimize the rapid release of the agent, it may be advantageous to heat the liposomes up to about60C to facilitate loading. To load the pharmaceutical agents into the liposomes utilizing the above-described treatment systems, the methods described in Mayer, et al. PCT Publication No. WO 88/06442, September 7, 1988, relvant portions of which are incorporated by reference, herein may be modified for use with the agents of the present invention.

In a liposome-drug delivery system, the pharmaceutical agent is entrapped in or associated with the liposome and then administered to the patient to be treated. As used throughout the specification, pharmaceutical agent, drug and agent are used interchangably. For example, see Rahman et al., U.S. Patent No.

3,993,754; Sears, U.S. Patent No. 4,145,410; Papahadjopoulos et al., U.S. Patent No. 4,235,871; Schneider, U.S. Patent No.

4,114,179; Lenk et al., U.S. Patent No. 4,522,803; and Fountain etal., U.S. Patent No. 4,588,578. In the present invention, any number of different pharmaceutical agents and different pharmaceutical types may be entrapped in or associated with liposomes. For example, pharmaceutical agents useful in the present invention may include any agent which readily passes through a liposomal layer(s) and exhibits limited solubility in a buffer solution internaltosthe liposome at an ion concentration or pH at which the pharmaceutical agent is in an ionized form.

Such agents may include antineoplastics; for example mitoxantrone, local anaesthetics; for example, lidocaine, dibucaine and chlorpromazine, bronchodilators; for example, metaproterenol, terbutaline and isoproterenol, beta-adrenergic blockers, for example propanolol, timolol and labetolol; antihypertensive agents, for example clonidine and hydralazine; anti-depressants, for example, imipramine, amitryptyline and doxepim, anti-convulsants, for example, phenytoin, anti-emetics, for example, procainamide and prochlorperazine; antihistamines, for example, diphenhydramine, chlorpheniramine and promethazine; anti-arrhythmic agents, for example, quinidine and disopyramide, anti-malarial agents, for example, chloroquine, quinacrine and quinine; and analgesics, among a number of additional pharmaceutical agents.

In general, internal buffers to be used in the liposomal compositions of the present invention are chosen using several criteria, the most important of which, after buffer strength, is the solubility characteristics of the pharmaceutical agent to be loaded in the buffer solution, as described hereinabove. It is preferred that the buffer system used as the internal buffer has a buffer strength of at least about 50 mM, preferably within the range of about 100 mM to about 300 mM, and most preferably about 300 mM.The most preferred buffer solutions for use as the internal buffer system of the present invention are therefore characterized by their inability to solubilize the ionized, preferably protonated pharmaceutical agent, i.e., the ionized pharmaceutical agent is generally soluble in the buffer solution to an extent no greater.than about 65 mMol, preferably no greater than about20mMol and most preferably no greater than about 10 mMol and which also have buffer strengths of at least about 50 mM, preferably about 100 to about 300 mM, most preferably about 300 mM. Most preferably, the internal buffer solution precipitates the ionized species of the pharmaceutical agent out of solution.

The choice of buffer to use as the internal buffer solution will vary depending upon the pharmaceutical agent chosen for loading. One of ordinary skill in the art will be able to assess the relative solubilities of ionized species of a pharmaceutical agent and the buffer strength to determine the buffer solution to be used as the internal buffer solution.

Any buffer solution having the characteristics generally described hereinabove may be used in the present invention, provided that the solution is pharmaceutically compatible, i.e., the solution may be administered to the patient without deleterious affects. Typical internal buffer solutions include citric acid, oxalic acid, succinic acid and other organic acid salts being preferred, among others. Citric acid in a concentration ranging from about 100 mM to about 300 mM is preferred. Most preferably, the citric acid buffer solution has a concentration ranging from about 100 mM to about 300 mM. Typical external buffer solutions may include NaCl, KCl, potassium phosphate, sodium bicarbonate, sodium carbonate, sodium bisphosphate, potassium sulfate and
HEPES, and mixtures thereof, among others.

Loading efficiencies of pharmaceutical agents utilizing the present invention generally range from about 20% up to about 100%, preferably at least about 50%. In general, the loading efficiencies for pharmaceutical agents according to the present invention are greater than expected from the Henderson-Hasselbach relationship. Of course, not all agents readily accumulate in liposomes according to the Henderson-Hasselbach relationship, and certain agents (see Table 1, Example 1) appear, in certain cases, not to accumulate at all. This phenomenon may be the result of the pharmaceutical agent being too polar for penetration of the liposomes, or other factors.Of course, one of ordinary skill in the art will recognize that to maximize the loading of a pharmaceutical agent into liposomes, it may be necessary to change the lipid constituents of the liposomes, or, in certain cases, to utilize ionophores or other agents which may enhance the penetration of the liposome by the agent in practicing the present invention.

The liposomes formed by the procedures of the present invention may be lyophilized or dehydrated at various stages of formation. For example, the lipid film may be lyophilized after removing the solvent and prior to adding the drug. Alternatively, the lipid-drug film may be lyophilized prior to hydrating the liposomes. Such dehydration may be carried out by exposure of the lipid or liposome to reduced pressure thereby removing all suspending solvent. The liposomes may be dehydrated in the presence of a hydrophilic agent according to the procedures of

Bally et al, PCT Publication No. 86/01102, published February 27, 1986, entitled "Encapsulation of Antineoplastic Agents inLiposomesn, Janoff et al., PCT Publication No. 86/01103, published February 27, 1986, entitled "Dehydrated Liposomes",
Schneider et al., in U.S.Patent No. 4,229,360, issued October 29, 1980 and Mayer, et al. PCT Publication No. 88/06442, published September 7, 1988, relevant portions of which are incorporated by reference herein. Alternatively or additionally, the hydrated lipsome preparation may also be dehydrated by placing it in surrounding medium in liquid nitrogen and freezing it prior to the dehydration step. Dehydration with prior freezing may be performed in the presence of one or more protective agents, such as sugars in the preparation according to the techniques of
Bally, et al., PCT Application No. 86/01103 published February 27, 1986, relevant portions of which are also incorporated by reference herein. Such techniques enhance the long-term storage and stability of hte preparations. For example, the pharmaceutical agent may be mixed with a sugar solution in a sugar: lipid weight/weight ratio ranging from about 0.5:1 to about 100:1, preferably about 20:1, without affecting the ability of the liposome to retain loaded agent during rehydration. In this aspect of the present invention, the liposomes preferably range in size from about 0.1 to about 0.2 microns.

In one preferred embodiment, the sugar is mannitol, or mannitol:glucose:lactose in a 2:1:1 w/w/w ratio. Following rehydration in distilled water, the preparation is preferably heated for ten minutes at an elevated temperature, for example 60'C. Other suitable methods may be used in the dehydration of the above-disclosed liposome preparations. The liposomes may also be dehydrated without prior freezing.

Once the liposomes have been dehydrated, they can be stored for extended periods of time until they are to be used.

The appropriate temperature for storage will depend on the lipid formulation of the liposomes and the temperature sensitivity of encapsulated materials. For example, various antineoplastic agents are heat labile, and thus dehydrated liposomes containing such agents should be stored under refrigerated conditions e.g.

at about4iC, so that the potency of the agent is not lost.

Also, for such agents, the dehydration process is preferably carried out at reduced temperatures, rather than at room temperature.

When the dehydrated liposomes are to be used, rehydration is accomplished by simply adding an aqueous solution, e.g., distilled water or an appropriate buffer, to the liposomes and allowing them to rehydrate. The liposomes can be resuspended into the aqueous solution by gentle swirling of the solution.

The rehydration can be performed at room temperature or at other temperatures appropriate to the composition of the liposomes'and their internal contents. If the antineoplastic agent which is to be administered was incorporated into the high drug to lipid ratio liposomes prior to dehydration, and no further composition changes are desired, the rehydrated liposomes can be used directly in the cancer therapy following known procedures for administering liposome encapsulated drugs. Alternatively, using the transmembrane pH gradient procedures described above, ionizable antineoplastic agents can be incorporated into the rehydrated liposomes just prior to administration.In connection with this approach, the concentration gradient used to generate the transmembrane pH gradient can be created either before dehydration or after rehydration using the external medium exchange techniques described above. For example, the high drug to lipid ratio liposomes may be dehydrated prior to establishing the transmembrane pH gradient, for example, dehydrated from their first external medium. Upon rehydration, the pH gradient can be established by admixing the liposomes with the second external medium of relatively acidic or basic pH. The antineoplastic agent can be admixed with the liposomes simultaneously with or following the establishment of the pH gradient.

In the case where the liposomes are dehydrated after having a transmembrane pH gradient, the liposomes may be rehydrated by admixing them with an aqueous solution of neutral pH.

For example, in the above-mentioned case where liposomes containing citric acid buffer as the first medium are used, the rehydration step would proceed by adding sodium carbonate and the pharmaceutical agent, for example, propanolol. Where the liposomes already contain the base (e.g. sodium carbonate), and therefore already have the transmembrane pH gradient are rehydrated, water or another neutral aqueous solution, and doxorubicin are added. Finally, in the case where liposomes having a transmembrane pH gradient and containing the pharmaceutical agent have been dehydrated, rehydration proceeds using water or another aqueous solution. Alternatively, a second pharmaceutical agent may be added, if desired.

Liposomes containing the pharmaceutical formulations of the present invention may be used therapeutically in mammals, especially humans, in the treatment of a number of disease states or pharmacological conditions which require sustained release formulations as well as repeated administration.

The mode of administration of the liposomes containing the pharmaceutical agents of the present invention may determine the sites and cells in the organism to which the compound may be delivered. The liposomes of the present invention may be administered alone but will generally be administered in admixture with a pharmaceutical carrier selected with regard to the intended route of administration and standard pharmaceutical practice. The preparations may be injected parenterally, for example, intravenously. For parenteral administration, they can be used, for example, in the form of a sterile aqueous solution which may contain other solutes, for example, enough salts or glucose to make the solution isotonic. The liposomes of the present invention may also be employed subcutaneously or intramuscularly.Other uses, depending upon the particular properties of the preparation, may be envisioned by those skilled in the art.

For the oral mode of administration, the liposomal for mulations of the present invention can be used in the form of tablets, capsules, losenges, troches, powders, syrups, elixirs, aqueous solutions and suspensions, and the like. In the case of tablets, carriers which can be used include lactose, sodium citrate and salts of phosphoric acid. various disintegrants such as starch, lubricating agents, and talc are commonly used in tablets. For oral administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents can be added.

For the topical mode of administration, the liposomal formulations of the present invention may be incorporated into dosage forms such as gels, oils, emulsions, and the like. These formulations may be administered by direct application as a cream, paste, ointment, gel, lotion or the like.

For administration to humans in the treatment of disease states or pharmacological conditions, the prescribing physician will ultimately determine the appropriate dosage of the neoplastic drug for a given human subject, and this can be expected to vary according to the age, weight and response of the individual as well as the pharmacokinetics of the agent used.

Also the nature and severity of the patient's disease state or pharmacological condition will influence the dosage regimen.

While it is expected that, in general, the dosage of the drug in liposomal form will be about that employed for the free drug, in some cases, it may be necessary to administer dosages outside these limits.

The following examples are given for purposes of illustration only and are not to be viewed as a limitation of the scope of the invention.

ExamPle 1
Preparation of LoadedLicosomes
The following pharmaceutical agents were loaded or attempted to be loaded into liposomes comprising egg phosphatidylcholine (EPC, from Avanti Polar Lipids, Inc.,
Birmingham, Alabama) or EPC and cholesterol 55:45 (molar ratio) (cholesterol from Sigma Chemicals, St. Louis, MO.): propanolol, timolol, dibucaine, chlorpromazine, lidocaine, quinidine, pilocarpine, physostigmine, dopamine, imipramine, diphenhydramine, quinine, chloroquine, quinacrine, dauorubicin, vincristine and vinblastine (obtained from Sigma Chemicals, St.

Louis, MO.), doxorubicin, epirubicin (obtained from Adria
Laboratories, Mississauga, Ont. Canada), mitoxantrone (obtained from Cyanamid Canada Inc., Montreal Que. (Canada), codeine and pethidine (obtained from Abbott Laboratories, Ltd. Downsview,
Ont. Canada). The radiolabels, 3Hdipalmitoylphosphatidylcholine, 14Cdipalmitoylphosphatidylcholine, 14C-dopamine and 14C-Imipramine were obtained from Amersham while 3H-chlorpromazine, 3Hpropranolol, 14C-pilocarpine, 14C-chlorpromazine, 14C-methylamine and 14C-lidocaine were obtained from New England Nuclear. The
Liposome Company, Inc. (Princeton, N.J.) kindly provided 14Ctimolol. Salts and reagents used were of analytical grade.

All loading of pharmaceutical agents were into EPC vesicles (containing 3H-Dipalmitoylphosphatidylcholine) or
EPC:cholesterol mixtures (55:45 molar ratio). EPC:cholesterol mixtures were prepared by colyophilization

from benzene:methanol (95:5 v/v). The dry lipid was hydrated with 300 mM citrate pH 4.0 as internal buffer solution and the resultant MLVs were subjected to five freeze-thaw cycles employing liquid nitrogen to enhance solute distribution according to Mayer, et al. Biochim.

Biochvs. Acta, 817, 193 (1986). Large unilamellar vesicles were then prepared using an Extruder (Lipex Biomembranes, Vancouver,
Canada) employing the LUVET procedure as described by Hope, et al. Biochim.Biochys. Acta, 812, 55, (1985) with 100 nm pore size polycarbonate filters (Nucleopore, Inc.). To establish a pH gradient the vesicles were then passed down a Sephadex G-50 (fine) colume (1.5 X 10cm) preequilibrated with 300 mM NaCl, 20 mM
HEPES, pH 7.5.

Large unilammelar vesicles (approximately 1 mM lipid) were incubated with the agent (0.2 mM) in 300 mM NaCl, 20 mM
HEPES, pH 7.5 at25 C. At various times up to 2 hours, aliquots (100 ul) of the mixture were taken and vesicles separated from unentrapped drug by centrifugation through a 1 ml'minicolumn" of
Sephadex G-50 (medium) as described by Pick, Arch. Biochem.

BioDhvs. 212, 186, 1981. Lipid and drug were quantified by the following procedure.

Lipid concentrations were determined by liquid scintillation counting of 3H-DPPC or 14C-DPPC using a Packard 2000 CA instrument. Similarly, pilocarpine, chlorpromazine, timolol, propranolol, imipramine, lidocaine and dopamine were quantified using tracer quantities of 3H- or 14C-radiolabel.

Physostigmine was assayed by fluorescence spectroscopy following solubilization of vesicles in 60% ethanol (v/v). The excitation and emission of wavelengths used were 305 and 350 nm, respectively. Quinacrine, chloroquine and quinine were also quantified from their fluorescence using excitation and emission wavelengths of 420 nm, 505 nm; 335 nm, 375 nm; and 335 nm, 365 nm; respectively.

Vinblastine and vincristine were assayed by U.V. spectroscopy from their absorbances at 262 nm and 297 nm, respectively, following solubilization of the vesicles in 80% ethanol.

Codeine was also measured by U.V. spectroscopy at 220 nm in this case after solubilization in 40 mMoctyl-beta-D-glucopyranoside.

Mitoxantrone was quantified from its absorbance at 670 nm following solubilization of the vesicles in 2% Triton-X100.

Diphenhydramine was assayed by gas-liquid chromatography using a HP 9850 gas chromatograph fitted with a Chromatographic
Specialties DB-225(25% cyanopropylphenyl) capillary column. The helium carrier flow rate was 1 ml/min and detection was by flame ionization. An internal standard, methylpentadecanoate, was used to quantify diphenhydramine following its extraction from the aqueous sample in diethylether and its separation from EPC by thin layer chromatography. Transbilayer pH gradients were quantified employing the weak base methylamine (14C-labelled) as previously described by Bally, et al., Chem.Phvs. Lipids, 47, 97, (1988).

The results of the loading experiment appear in Table 1, below. Basically, the loading of liposomes with the agents described above may be defined on the basis of their uptake char acteristics. Four drug categories may be defined based upon their uptake characteristics.

The first category of pharmaceutical agents exhibited complete, stable uptake. Propanolol, dopamine, dauonorubicin, epirubicin, dibucaine, imipramine and doxorubicin exhibited the characteristics of this drug category. All of the drugs within this category exhibited uptake greater than predicted from theHenderson/Hasselbach equation. The accumulation of mitoxantrone by EPC liposomes exhibiting a proton gradient is shown in Figure 1.

The second category showed partial, but stable uptake.

Timolol, lidocaine, chlorpromazine, serotonin and chloroquine exhibited the characteristics of this drug category. Timolol was loaded to the extent of about 100 nmoles/umole lipid (about 50% of available drug, see figure 2A) and quinacrine was loaded to a level of about 80 nmoles/umole lipid after 30 minutes (see figure 2B). While accumulation is lower than in the first group of agents, nevertheless uptake is quite substantial. In the case of timolol, an internal concentration of about 65 mM is achieved against an external concentration of 100 uM.

The third category shows a partial uptake followed by a rapid release of agent from the liposome. Figure 3 indicates a rapid virtually complete accumulation of quinidine into the vesicles and within 30 minutes about 50% of the agent has leaked back out of the vesicles (Figure 3A). Other agents which leak back out of EPC vesicles include quinine, diphenhydramine, vinblastine and vincristine. The leakage rates vary considerably with vincristine and vinblastine loaded vesicles losing only 27% of initially sequestered drug overtwohours. This loss is associated with a corresponding reduction in residual change in pH as determined using methylamine. A similar decrease in proton gradient is observed as quinine and diphenhydramine are released from EPC vesicles.

The fourth category of pharmaceutical agents, physostigmine, codeine and pilocarpine exhibited no measurable response to the transmembrane pH gradient. The suggestion that these agents cause a major increase in membrane permeability resulting in loss of ion gradient is not borne by the date from physostigmine (Figure 4). Under the conditions used to assess uptake of physostigmine (200 uM) only a small decrease in measured change in pH was observed.

Table 1
Extent and Stability of accumulation of Various Drugs
Vesicles Exhibiting a pH Gradient
Drug Class Uptake 15 Minutes Uptake 2 Hours
(nmoles/umoles lipid) (nm/um lipid)Cateaoro 1
Mitoxantrone Antineoplastic 200 198
Epirubicin Antineoplastic 201 200
Daunorubicin Antineoplastic 200 204
Doxorubicin Antineoplastic 202 203
Dibucaine Local Anaesthetics 194 176
Propanalol Adrenergic 198 187
Dopamine Biogenic Amine 1901 177Cateaorv 2
Timolol Adrenergic 95 97
Lidocaine Local Anaesthetic 87 87
Chlorpromazine Local Anaesthetic 98 96
Serotonin Biogenic Amine 802 78Chloroquine Antimalarial 1043 88
Quinacrine Antiprotozoal 731 71Cateaorv 3
Quinidine Antiarrhythmic 203 74
Quinine Antimalarial 1483 81
Diphenhydramine Antihistamine 1763 87
Vinblastine Antineoplastic 1753 127
Vincristine Antineoplastic 178 130Category 4
Codeine Analgesic < 1 < 1
Pilocarpine Cholinergic < 1 < 1
PhysostigmineCholinergic < 2 < 1
Footnote:1, maximum uptake at 30 minutes,, maximum uptake at
90 minutes,3,.maximum uptake at 5 minutes
ExamPle 2
Comparison Between Level ofDrua Uttake and
theDrug's Octanol: Water Partition Coefficient
Levels of drug uptake were compared to their octanol/water partition coefficients to determine the extent that partition coefficient and the possibility that an agent was partitioning into the liposome bilayer might determine the extent of uptake of a pharmaceutical agent.From Table 2, below, it appears that no clear relationship exists between drug uptake and its partition coefficient. Although the values given may not accurately reflect membrane/water partition coefficients, they are merely being used for a comparative basis. Chlorpromazine and doxorubicin, for example, have similar partition coefficients yet display very different uptake levels (98 vs 202). On the other hand, timolol and chlorpromazine are accumulated by vesicles to a similar extent despite a large difference in their partition coefficients. While partition coefficient for a ionized drug may influence drug uptake, it can not be taken to explain the differences between the agents studied.

Table 2
A Comparison Between the Level of Drug Uptake and
Its Octanol: Water Partition Coefficient
Drug Maximum Uptake Log Octanol:Water
nmoles/umole lipid Partition Coefficient1
Daunorubicin 200 3.5
Doxorubicin 202 , 1.1
Vincristine 178 2.8
Chlorpromazine 98 1.5

Dibucaine 194 4.4
Propranolol2 198 1.3
Timolol2 95 -0.1
Physostigmine 00.2
Imipramine 182 4.6
Diphenhydramine 176 3.4
Quinine 148 1.7
Codeine 0 1.2
Footnotes I All data taken from Leo, A., et al. Chem. Rev., 71,
525(1971) except for propranolol and timolol.

-From Merbate, L. G., et al.,Biophys. J., 49, 91,
(1986).

Example3
Comparison Between Level ofDrua UPtake and
theDrug's Solubilitv in Buffer Solution
Besides buffer strength, the factor that influences the level of drug uptake to the greatest extent is the solubility of the protonated species in the internal buffer. When the concentration of protonated drug inside the vesicle exceeds its solubility product and precipitation occurs this will effectively reduce the transmembrane concentration gradient for the remaining solube fraction thus allowing further accumulation by the vesicles. In table 3 is shown the maximum apparent solubilities in300mM citrate buffer, pH 5.0 for most of the drugs whose proton gradient dependent uptake was examined.Drugs such as mitoxantrone, epirubicin, doxorubicin and daunorubicin which exhibit complete and stable uptake are relatively insoluble in the intravesicular medium. This indicates that most of the accumulated drug is in the form of a precipitate and does not contribute to the concentration gradient of the soluble protonated species, thus accounting for the-high levels of uptake observed. In addition, if most of the intravesicular drug is precipitated the concentration of free drug available to partition into the membrane is correspondingly reduced which will contribute to the observed stability of the transmembrane proton gradient.As expected, agents such as timolol, lidocaine, quinacrine and chloroquine which exhibit uptake in good agreement with the Henderson-Hasselbach equation have apparent solubilities which are in excess of the intravesicular concentrations achieved (See table 3, below). Without being bound by any theory, the solubility data may explain most of the observed differences in uptake characteristics for the various drugs examined. The data indicates that solubility data is most important in determining uptake of drugs into liposomes.

We note that dibucaine, propranolol and dopamine may also be loaded in liposomes in an amount significantly greater than predicted by the Henderson-Hasselbach equation. This is a surprising result considering that the three agents' apparent solubility is greater than the final internal concentration of the agent in the liposome.

Table 3
Apparent Maximum Drug Solubility in 300mN
Citrate Buffer, pH 5.0
Drug Apparent Maximum Solubility
(mM)
Mitoxantrone < 0.01
Epirubicin 0.26
Daunorubicin 9.10
Doxorubicin 0.24
Vincristine > 35
Vinblastine 19.1
Lidocaine 240
Dibucaine > 700
Propanolol
Timolol 135Quinine
Dopamine 1400
Quinine 1.05
Chloroquine 585
Quinacrine 90
Example 4
PreDaration of LiDosomes Loaded with
Isoproterenol. Metaproterenol and Terbutaline
Egg phosphatidylcholine (EPC) purchased from Avanti Polar
Lipids (Birmingham, Alabama), and 14C-methylamine was purchased from New England Nuclear. All

other chemicals and buffers were purchased from Sigma (St. Louis, MO.) and were used without purification.

Large Unilamellar Vesicles(lugs) were produced by extrusion according to the method of Hope, et al., Biochim.Biothys.

Acta, 817, 193 (1985) Briefly, LUVs were produced by extrusion of frozen and thawed lipid dispersions prepared in 300 mM citrate, pH 4.0, through 0.1 or 0.2 um polycarbonate filters (Nucleopore) employing an extrusion device (Lipex Biomembranes,
Vancouver, Canada). Vesicles prepared by this technique employing 0.1 um filters have trapped volumes of 1.5 uL/umole phospholipid as determined using 14C or 22Na and have an average diameter of 90 nm. Phospholipid concentrations were determined by assay of lipid phosphorous as previously described by Fiske and Subbarrow, J. Biol. Chem., 66, 375, (1925). Transmembrane pH gradients were established according to Example 1, and untrapped internal buffer removed by passing theLUVs down a SephadexG-50 column equilibrated with the external buffer [150 mM NaCl, 20 mM
HEPES, pH 7.4]. Induced pH gradients were determined by measuring the transmembrane distributions of 14C-methylamine as described by Hope, et al., supra. In short, methylamine was added to the vesicle system to a final concentration of 0.5 uCi/mL. At appropriate times, aliquots (100 uL)were removed and passed down 1 mL Sephadex G-50 mini-columns as previously described. The trapped probe was determined by liquid scintillation counting employing a Packard 2000CA liquid scintillation counter, and phospholipid concentrations were determined. Transmembrane pH gradients were calculated according to the equation
change in pH=log[MeAm]i/[MeAm]O.

The bronchodilators, isoproterenol, metaproterenol and terbutaline were incubated with liposomes with a transmembranepH gradient prepared as above at the indicted temperatures in a 150 mMNaC1, 20 mM HEPES, pH 7.4 buffer containing 500 uM of the bronchodilator and 6 mM of the phospholipid. Control samples without a transmembrane pH gradient were incubated at pH 4.0 or 7.4 (both inside and outside the vesicles) to determine the degree of gradient-independent membrane binding. The pH 4.0 control consisted of the vesicles prepared as above, but the external buffer was 150 mMNàCl, 20 mM citrate, pH 4.0. For the pH 7.4 control, the vesicles were prepared with 150 mMNaCl, 20 mM HEPES pH 7.4, both internally and externally.

Free drug was separated from vesicles using 1 mL SephadexG-50 minicolumns as described above and assayed (photometrically). Figure 5 shows the entrapment of the bronchodilators metaproterenol, terbutaline and isoproterenol in response to pH gradients using (EPC) 200 nmextruded liposomes. Vesicles containing 300 mM citrate, pH 4.0 were incubated with a 500 uM drug solution at pH 7.4. The liposomes accumulate the drug to levels of greater than 60 nmoles/umole lipid. This is roughly 70% trapping efficiency, and corresponds to an inside:outside drug concentration ratio of about 190:1, which assumes an internal volume of 2.2 uL/umole lipid. If all drugiso in the aqueous space of the vesicles, an internal drug concentration of about 30 mM is calculated.Uptake is stable for at least 4 hours and is complete within minutes. In the absence of the transmembrane pH gradient, background binding of these drugs is less than 15 nmoles/umole lipid for both pH 4.0 (inside and outside the vesicles) and pH 7.4 (in and out), indicating that non-specific binding to the lipid is not responsible for the association of these drugs with the liposomes, and that the association is a function of the proton gradient rather than the absolute pH.

ExamPle 5
Effect of Drua Uptake on ResidualpH Gradient
As Measured bvMethyl amine Distribution
The logarithm of the ratios of the internal and external concentrations of the radioactive methylamine can be used to measure the transmembrane change in pH, because the methylamine probe does not dissipate the internal proton poool at these concentrations. When the internal and external pH is 7.4 or 4.0 (no gradient) the methylamine does not detect any gradient (Figure 6). When vesicles with an internal pH of 4.0 are incubated in a pH of 7.4, the methylamine distribution indicates a 3.0 unit pH gradient, in good agreement with the 3.4 pH unit gradient. When metaproterenol is added to the external buffer to a final concentration of 500 uM, the gradient dissipates to about 2.3 pH units as the drug is accumulated.The date indicates that the 190 fold achieved by the drug approximates the residual proton gradient (pH of 2.3 units).

Example 6
Effect of Heat on Entrapment of Metaproterenol
The rate of metaproterenol entrapment is increased by increased temperature (See Figure 7), reaching steady-state levels after 2 hours at21 C, but faster than 15 minutes when incubated at60'C, The extent of drug uptake is not dramatically affected by the temperature of incubation.

Example 7
Effect of Cholesterol on Entrapment of Bronchodilators
The inclusion of cholesterol in liposomes on the uptake of bronchodilators was investigated. Cholesterol decreased both the rate of uptake, and the total amount of trapped drug per umole of lipid. Since the internal trapped volume of
EPC:cholesterol (55:45 mole %) determined using 14C inulin and 22Na is about 30% lower than vesicles composed of EPCalone, the actual concentration gradients of metaproterenol achieved is similar in both cases. See Figure 8.

ExamDle 8
Effect ofDrua to Lipid Ratio on the Amount ofTrapped Drua
The amount of entrapped drug also depends on the initial drug to lipid ratio (see Figure 9). At low drug to lipid ratios, the amount of drug entrapped by the vesicles reflects the imposed transmembrane pH gradient greater than 3 units. At sufficiently high drug concentration, the reionization of the drug in the vesicle interior causes a decrease in the internal pH (Figure 6).

Equilibrium levels of drug entrapment would therefore be expected to relect the final change in pH rather than the initial change in pH as the ionized drug overwhelms the internal buffering capacity of the vesicles.

Example 9
Effect of Buffer Strength On Entrapment
Extent of the buffer capacity (buffer strength) is a factor which affects the ability of the liposome to trap ionized drug. Low internal buffer capacity affects the extent of drug accumulation. Below 100 mM citrate, the extent of drug accumulation is indeed affected by the internal citrate concentration (Figure 10). Under these conditions, re-ionization of the drug overwhelms the internal buffering capacity of the vesicle interior, raising the pH of the vesicles and dissipating the change in pH (inset). Including greater levels of citrate than 300 mM in the vesicles does not dramatically increase the levels of trapped drug, since there is still a large residual pH gradient in these cases and the internal buffering capacity does not limit drug uptake.

It will be understood by those skilled in the art that the foregoing description and examples are illustrative of practicing the present invention, but are in no way limiting. Variations of the detail presented herein may be made without departing from the spirit and scope of the present invention.

ACCUMULATION OF DRUGS INTO LIPOSOMES BY A PROTON GRADIENT

Claims of corresponding document: **WO9014105**

Claims:

1. A liposome composition comprising:
a liposome having a transmembrane ion gradient comprising at least one pharmaceutical agent selected from the group consisting of local anaesthetics, bronchodilators, beta-adrenergic blockers, anti-hypertensive agents, anti-depressants, anticonvulsants, anti-emetic agents., anti-histamines, anti-arrhythmic agents, anti-malarial agents and analgesics;
at least one lipid;
a first internal aqueous buffer solution;
and a second external aqueous buffer solution, said pharmaceutical agent having a solubility less than a concentration of said agent in said internal buffer solution to produce an amount of pharmaceutical agent in said liposome greater than expected from the transmembrane ion gradient.

2. The composition according to claim 1 wherein said ion gradient is a pH gradient.

3. The composition according to claim 1 wherein said pharmaceutical agent has a solubility in said internal buffer solution of less than about 20 mM and said agent has a solubility in said external buffer solution of at least about 0.2 mM.

4. The composition according to claim 3 wherein said pharmaceutical agent has a solubility in said internal buffer solution of less about than about 10 mM.

5. The composition according to claim 2 wherein said pharmaceutical agent is selected from the group consisting of dopamine, dibucaine, chlorpromazine, lidocaine, serotonin, quinacrine, metaproterenol, terbutaline, isoproterenol, quinidine, quinine, diphenhydramine and chloroquine.

6. The composition according to claim 3 wherein said pharmaceutical agent is selected from the group consisting of dibucaine, dopamine, quinidine, imipramine and diphenhydramine.

7. The composition according to claim 2 wherein said lipid is selected from the group consisting of phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cardiolipin and a mixture of phosphatidylethanolamine and phosphatidylcholine in a weight ratio of phosphatidylethanolamine to phosphatidylcholine of about 30:70 to about 45:55.

8. The liposome composition according to claim 1 which is dehydrated.

9. A pharmaceutical composition comprising the liposome composition according to claim 1 and a pharmaceutcally acceptable carrier or diluent.

10. A liposome composition comprising:
a liposome having a transmembrane pH gradient comprising at least one pharmaceutical agent selected from the group consisting of metaproterenol, terbutaline and isoproterenol;
at least one lipid;
and at least one aqueous buffer solution; wherein said pharmaceutical agent accumulates in said liposome in an amount equal to about 70% entrapment efficiency.

11. The composition according to claim 10 wherein said lipid is selected from the group consisting of phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cardiolipin and a mixture of phosphatidylethanolamine and phosphatidylcholine in a weight ratio of phosphatidylethanolamine to phosphatidylcholine of about 30:70.to about 45:55.

12. The composition according to claim 10 wherein said lipid is phosphatidylcholine.

13. The composition according to claim 10 wherein said buffer solution is a buffer combination comprising an internal aqueous buffer solution and an external aqueous buffer solution.

'14. The composition according to claim 10 wherein said first buffer solution is selected from one or more of the group consisting of citric acid, oxalic acid, succinic acid and salts of organic acids and wherein said

second buffer solution is selected from the group consisting of sodium chloride, potassium chloride, potassium phosphate, sodium bicarbonate, sodium carbonate, sodium bisphosphate, potassium phosphate, potassium sulfate and HEPES.

15. The composition according to claim 10 wherein said buffer solution is a buffer combination comprising a first internal buffer solution comprising citrate buffer and a second external buffer solution comprising a mixture of NaCl and HEPES.

16. The composition according to claim 15 wherein said first internal buffer solution is a citrate buffer of concentration ranging from about 100 mM to about 300 mM and said second buffer contains NaCl and HEPES, said NaCl ranging in concentration from about 100 to 400 mM and said HEPES ranging in concentration from about 10 mM to about 30 mM.

17. The liposome composition according to claim 10 which is dehydrated.

18. A pharmaceutical composition comprising the liposome composition according to claim 10 and a pharmaceutcally accep table carrier or diluent.

19. A liposome composition comprising:
a liposome having a transmembrane ion gradient comprising at least one pharmaceutical agent selected from the group consisting of quinine, diphenhydramine and quinidine which rapidly releases from said liposome after accumulation;
at least one lipid which prevents said pharmaceutical agent from rapidly releasing from said liposome after accumulation;
and an aqueous buffer solution.

20. The composition according to claim 19 wherein said ion gradient is a pH gradient.

21. The composition according to claim 19 wherein said lipid is a lipid combination comprising at least one first lipid selected from the group consisting of phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cardiolipin and a mixture of phosphatidylethanolamine and phosphatidylcholine in a weight ratio of phosphatidylethanolamine to phosphatidylcholine of about 30:70 to about 45:55 and at least one second lipid comprising a membrane-stabilizing lipid component.

22. The composition according to claim 21 wherein said membrane-stabilizing component is cholesterol.

23. The composition according to claim 22 wherein said first lipid is phosphatidylcholine and said second lipid is cholesterol and wherein said weight ratio of phosphatidylcholine to cholesterol is about 70:30 to about 55:45.

24. The composition according to claim 23 wherein said weight ratio of phosphatidylcholine to cholesterol is about 55:45.

25. The liposome composition according to claim 19 which is dehydrated.

26. A pharmaceutical composition comprising the liposome composition according to claim 19 and a pharmaceutcally acceptable carrier or diluent.

27. A liposome composition comprising:
a liposome having a transmembrane ion gradient comprising at least one pharmaceutical agent selected from the group consisting of quinine, diphenhydramine and quinidine which rapidly releases from said liposome after accumulation;
at least one lipid which prevents said pharmaceutical agent from rapidly releasing from said liposome after accumulation;
a first internal aqueous buffer solution;
and a second external aqueous buffer solution, said pharmaceutical agent having a solubility less than a concentration of said agent in said internal buffer solution to produce an amount of agent in said liposome greater than expected from the transmembrane ion gradient.

28. The composition according to claim 27 wherein said ion gradient is a pH gradient.

29. The composition according to claim 27 wherein said lipid is a lipid combination comprising at least one lipid selected from the group consisting of phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cardiolipin and a mixture of phosphatidylethanolamine and phosphatidylcholine in a weight ratio of phosphatidylethanolamine to phosphatidylcholine of about 30:70 to about 45:55 and at least one

membrane-stabilizing lipid component.

30. The composition according to claim 28 wherein said membrane-stabilizing lipid component is cholesterol.

31. The composition according to claim 28 wherein said first lipid is phosphatidylcholine and said membrane-stabilizing lipid component is cholesterol wherein said weight ratio of phosphatidylcholine to cholesterol is about 70:30 to about 55:45.

32. The composition according to claim 29 wherein said weight ratio of phosphatidylcholine to cholesterol is about 55:45.

33. The composition according to claim 27 wherein said pharmaceutical agent has a solubility in said internal buffer solution of less than about 20 mM and said agent has a solubility in said external buffer solution of at least about 0.2 mM.

34. The composition according to claim 27 wherein said pharmaceutical agent has a solubility in said internal buffer solution of less about than about 10 mM.

35. The composition according to claim 27 wherein said internal buffer solution has a buffer strength of at least about 50 mM.

36. The composition according to claim 27 wherein said internal buffer solution has a buffer strength ranging from about 100 to about 300 mM.

37. The composition according to claim 27 wherein said internal buffer solution has a buffer strength of about 300 mM.

38. The liposome composition according to claim 27 which is dehydrated.

39. A pharmaceutical composition comprising the liposome composition according to claim 27 and a pharmaceutically acceptable carrier or diluent.

40. A liposomal composition comprising:
a liposome having a transmembrane ion gradient comprising at least one pharmaceutical agent selected from the group consisting of dibucaine, propranolol and dopamine;
at least one lipid;
a first internal aqueous buffer solution;
and a second external aqueous buffer solution, said pharmaceutical agent accumulating in said liposome in an amount greater than expected from the transmembrane ion gradient.

41. The liposomal composition according to claim 40 wherein said lipid is egg phosphatidylcholine.

42. The liposomal composition according to claim 41 wherein said buffer is citric acid buffer ranging in concentration between about 100 and 300 mM.
